MyD88-dependent and -independent signaling by IL-1 in neurons probed by bifunctional Toll/IL-1 receptor domain/BB-loop mimetics

Christopher N. Davis*, Enrique Mann†, M. Margarita Behrens*, Svetlana Gaidarova*, Mitra Rebek*, Julius Rebek, Jr.†‡, and Tamas Bartfai*

*The Harold L. Dorris Neurological Institute and Department of Molecular and Integrative Neurosciences and †The Skaggs Institute of Chemical Biology and Department of Chemistry, The Scripps Research Institute, 10550 North Torrey Pines Road, La Jolla, CA 92037

Contributed by Julius Rebek, Jr., December 28, 2005

Interleukin (IL)-1β is a pluripotent proinflammatory cytokine that signals through the type-I IL-1 receptor (IL-1RI), a member of the Toll-like receptor family. In hypothalamic neurons, binding of IL-1β to IL-1RI mediates transcription-dependent changes that depend on the recruitment of the cytosolic adaptor protein myeloid differentiation primary-response protein 88 (MyD88) to the IL-1RI/IL-1 receptor accessory protein (IL-1RAcP) complex through homomeric Toll/IL-1 receptor (TIR)–TIR interactions. Through design and synthesis of bifunctional TIR mimetics that disrupt the interaction of MyD88 with the IL-1RI/IL-1RAcP complex, we analyzed the involvement of MyD88 in the signaling of IL-1β in anterior hypothalamic neurons. We show here that IL-1β-mediated activation of the protein tyrosine kinase Src depended on a MyD88 interaction with the IL-1RI/IL-1RAcP complex. The activation of the protein kinase Akt/PKB depended on the recruitment of the p85 subunit of PI3K to IL-1RI and independent of MyD88 association with the IL-1RI/IL-1RAcP complex. These bifunctional TIR–TIR mimetics represent a class of low-molecular-weight compounds with both an antiinflammatory and neuroprotective potential. These compounds have the potential to inhibit the MyD88-dependent proinflammatory actions of IL-1β, while permitting the potential neuronal survival supporting actions mediated by the MyD88-independent activation of the protein kinase Akt.

cytokine | interleukin | protein–protein interaction | inflammation

Interleukin (IL)-1β is a proinflammatory cytokine (1, 2) known to mediate a variety of host responses to infection and inflammation. These effects include components of the acute phase response such as induction of the elevation of body temperature (fever), anorexia, and somnolence through effects on non-rapid-eye-movement sleep (3–5). The concentration of this cytokine and its receptor are highly modulated during CNS injury, neurodegeneration, and inflammation (6). Chronic action of IL-1β in the brain leads to induction of additional inflammatory mediators, such as IL-6 and TNFα, and to the induction of biosynthetic enzymes for inflammatory mediators including cyclooxygenase-2 (COX2) and inducible NO synthase (7). Upon binding of IL-1β, the type-I IL-1 receptor (IL-1RI) forms a signaling heterodimer with the IL-1 receptor accessory protein (IL-1RAcP) (8). Both IL-1RI and IL-1RAcP belong to the family of Toll-like receptor proteins (9, 10), which transduce signaling through interactions with other Toll/IL-1 receptor (TIR) domain-containing proteins. Upon IL-1β binding, the cytosolic TIR-domain adaptor protein myeloid differentiation primary response protein 88 (MyD88) associates with both the IL-1RI and IL-1RAcP, triggering the recruitment of a series of signaling proteins (11, 12), leading to the activation of mitogen-activated protein kinases (13) and, ultimately, to the activation of the transcription factor NF-κB (14, 15).

We have shown previously with the use of a synthetic low-molecular-weight MyD88 mimetic, hydrocinnamoyl-L-valyl pyrrolidine (AS-1), that disruption of the interaction of MyD88 with the IL-1RI/IL-1RAcP complex prevents the development of the fever response induced by IL-1β in mice (16). These data support the hypothesis that the transcription-dependent effects (induction of COX2 and prostaglandin E_2) induced by IL-1β actions in the anterior hypothalamus (AH) require MyD88, similar to IL-1β-mediated effects in macrophages (17, 18).

We prepared a novel series of bifunctional TIR domain mimetics that were used on primary cultures of preoptic area (POA)/AH neurons (obtained from wild-type and MyD88$^{-/-}$ mice) for the analysis of the rapid effects of IL-1β exposure on two main signaling kinases responsible for the regulation of ion channels in neurons, the protein tyrosine kinase Src and the serine/threonine kinase Akt/PKB. We show that Src activation depends on the recruitment of the adaptor protein MyD88 to the IL-1RI/IL-1RAcP receptor complex in POA/AH neurons. The activation of Akt/PKB depended on PI3-kinase activation, through binding of its p85 subunit to IL-1RI, but independent of MyD88 association. The clear separation of the two pathways activated by IL-1β in POA/AH neurons observed in the presence of the MyD88 mimetics suggests that these compounds may have relevant antiinflammatory actions and promote neuronal survival in the nervous system.

PHARMACOLOGY

Results

Synthesis of TIR/BB-Loop Mimetics. Earlier, we reported the synthesis and pharmacological effects of a low-molecular-weight MyD88 mimetic AS-1 (data not shown), modeled on a tripeptide sequence of the BB-loop [(F/Y)–(V/L/I)–(P/G)] of the TIR domain (16). In an attempt to improve the affinity and specificity of AS-1, further chemical modifications have been made resulting in small-molecule inhibitors of TIR–TIR interactions. Removal of the butoxycarbonyl-protecting group of compound **1** by treatment with trifluoroacetic acid in dichloromethane (CH_2Cl_2) afforded the corresponding amine as the trifluoroacetate salt, which was monoalkylated by a reaction with 1-(3-bromopropyl) benzene in dimethylformamide in the presence of potassium carbonate, yielding amine **2**. Reaction of 2 eq of compound **2** with terephthaloyl chloride afforded dimer **3** (EM77) as a crystalline solid. In the same way, reaction of 2 eq of amine **2** with the diacid chloride of 2,5-pyridinedicarboxilic acid gave compound **4** (EM110) as a crystalline solid. Recrystalization of compounds EM77 and EM110 from ethyl acetate yielded clear crystals. The reaction scheme and solid-state structures are shown in Fig. 1.

Conflict of interest statement: No conflicts declared.

Abbreviations: IL, interleukin; COX2, cyclooxygenase-2; TIR, Toll/IL-1 receptor; AH, anterior hypothalamus; ESI, electrospray ionization; AS-1, hydrocinnamoyl-L-valyl pyrrolidine; MyD88, myeloid differentiation primary-response protein 88; IL-1RI, type-I IL-1 receptor; IL-1RAcP, IL-1RI/IL-1 receptor accessory.

‡To whom correspondence should be addressed. E-mail: jrebek@scripps.edu.

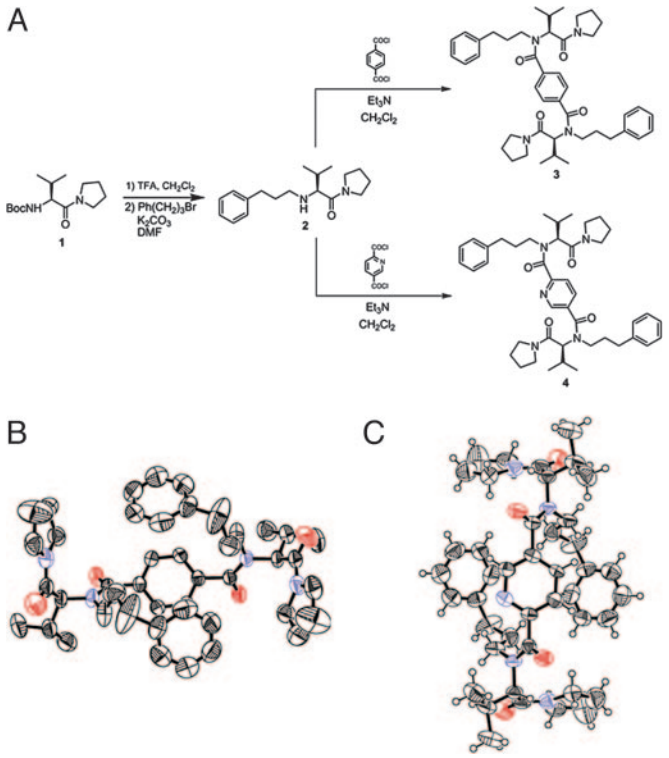

Fig. 1. Synthesis and crystal structure of TIR/BB-loop mimetics in Ortep presentation (30% occupation thermal ellipsoids). (*A*) Synthesis of BB-loop mimetic based on the [(F/Y)–(V/L/I)–(P/G)] consensus sequence in TIR domains of TLRs, IL-1RI, and MyD88. Compound **1**, commercial butoxycarbonyl-L-valine hydroxysuccinimide ester; Compound **2**, AS-1 (*B*) Compound **3**, EM77. (C) Compound **4**, EM110.

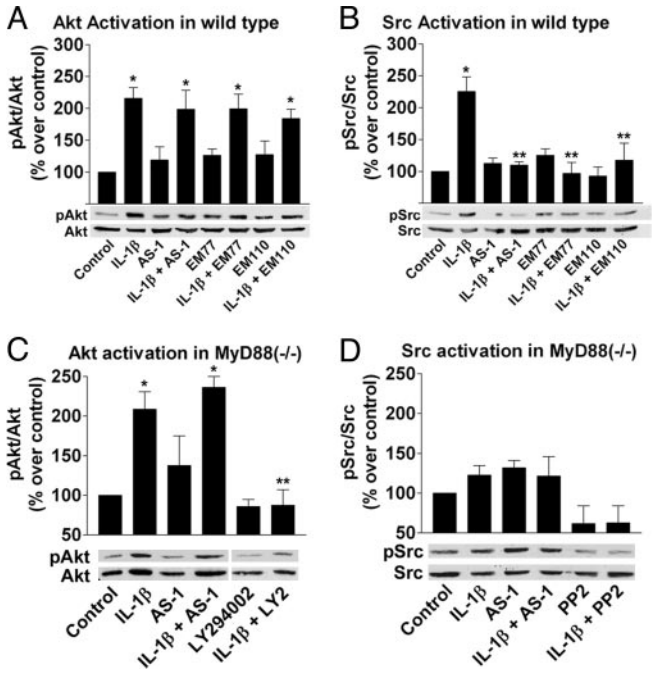

Fig. 2. IL-1β-mediated activation of Src, but not Akt, depends on the recruitment of MyD88. Representative Western blots are shown, and bar graphs indicate means ± SEM from at least three independent experiments. (*A* and *B*) Cultured wild-type AH neurons were developed on a preformed bed of glia from IL-1RI$^{-/-}$ mice. Neurons were stimulated with IL-1β (10–12 nM) for 10 min after a 15-min exposure to the TIR domain mimetics AS-1 (20 μM), EM77 (20 μM), or EM110 (20 μM). The IL-1β stimulations in the presence of drug in *A* were not statistically significant compared with IL-β treatment ($P > 0.05$). (*C* and *D*) Cultured AH neurons from MyD88$^{-/-}$ mice were developed on a preformed bed of glia from IL-1RI$^{-/-}$ mice. Neurons were stimulated with IL-1β (10–12 nM) for 10 min after a 15-min exposure to the MyD88 mimetic AS-1 (100 μM) and the PI3-kinase inhibitor LY294002 (20 μM) (*C*) or the Src-family inhibitor PP2 (2 μM) (*D*). Statistical significance was determined by ANOVA followed by Tukey's test; significance is indicated with an * compared with control and a ** compared with IL-1β treatment at $P < 0.05$.

Biological Effects of TIR/BB-Loop Mimetics. IL-1RI receptors are expressed in both neurons and glia in the AH as well as in AH cultures. We cultured AH neurons on top of a preformed glial bed obtained from IL-1RI$^{-/-}$ mice. In these mosaic cultures, no glial fibrillary acidic protein/IL-1RI double-labeled cells were found. The entire population of IL-1RI-expressing cells is purely neuronal, and responses to IL-1β are in the neurons alone. Neurons in this mosaic culture system continued to express the neuronal marker mitogen-activated protein-2 and IL-1RI, and electrophysiological studies on IL-1RI-mediated responses were indistinguishable from neurons in a wild-type culture (data not shown). The mosaic cultures made it possible to biochemically characterize the neuronal effects of IL-RI/MyD88 interactions in isolation from IL-1β-induced events in glia.

Using this mosaic culture system, we analyzed the IL-1β-mediated neuronal changes in the phosphorylation state of two signaling kinases, Akt and Src, known to be activated upon IL-1β binding to its receptor in nonneuronal systems (19–21). Using phospho-specific antibodies developed against the activated forms of Akt (phosphorylated at Ser-473) and Src (phosphorylated at Tyr-416), we demonstrated that exposure to IL-1β induced a rapid concentration and time-dependent phosphorylation of Akt and Src in AH neurons, with activation peaking from 5 to 10 min (Fig. 2 *A* and *B*; data not shown). To analyze whether the IL-1β-mediated Akt and Src activation required the recruitment of the cytosolic adaptor protein MyD88, we used the TIR domain mimetics AS-1 (shown to disrupt IL-1RI-mediated signaling *in vitro* and *in vivo* in ref. 16) and the two unique bifunctional analogs of AS-1, EM77 and EM110. The IL-1β-mediated increase in Akt phosphorylation was not prevented by preincubation of AH cultures with any of the MyD88 mimetics (20 μM; 15-min preexposure) (Fig. 2*A*), whereas the IL-1β-mediated Src activation required the recruitment of the cytosolic adaptor protein MyD88 because AS-1 and its bifunctional analogs all blocked the IL-1β-mediated increase in Src phosphorylation (Fig. 2*B*).

To confirm the lack of effect of AS-1 on Akt activation and the requirement of MyD88 for IL-1β-mediated Src activation, we cultured neurons from knockout mice lacking the expression of MyD88 (22). Cultured AH neurons from MyD88$^{-/-}$ mice were seeded on IL-1R$^{-/-}$ glia and allowed to develop to maturity. Exposure of these cultures to IL-1β induced a significant increase in the phosphorylation of Akt but not Src (Fig. 2 *C* and *D*, respectively). These data, taken together with the lack of effects of monofunctional and bifunctional TIR domain mimetics, indicate that MyD88 is not necessary for the activation of Akt in AH neurons but is necessary for the activation of Src.

To analyze whether PI3-kinase activation was the upstream mechanism of Akt activation in AH neurons, we exposed the cultures to the specific PI3-kinase inhibitor LY294002. Activation of Akt was prevented by a 15-min exposure to LY294002 (20 μM) (Fig. 3). The specificity of the PI3-kinase family inhibitor LY294002 is well documented (23, 24) and, at this concentration, was found not to have any effects on Src phosphorylation (data not shown).

The recruitment of PI3-kinase to the IL-IRI/IL-1RAcP complex

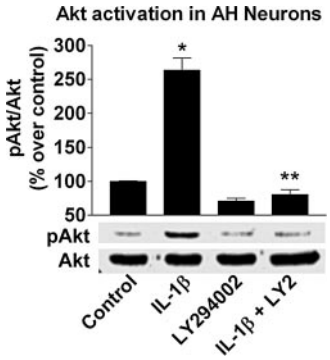

Fig. 3. IL-1β-mediated activation of Akt is blocked by LY294002. Representative Western blots are shown, and bar graphs indicate means ± SEM from at least three independent experiments. Cultured AH neurons from wild-type mice were developed on a preformed bed of glia from IL-1RI$^{-/-}$ mice. Neurons were stimulated with IL-1β (10–12 nM) for 10 min in the absence or presence of the PI3-kinase inhibitor LY294002 (20 μM). Statistical significance was determined by ANOVA followed by Tukey's test, and significance is indicated with an * compared with control and a ** compared with IL-1β treatment at $P < 0.05$.

has been shown to occur in nonneuronal systems through direct interaction of the p85 subunit of PI3-kinase to the IL-1RI (19, 25, 26). To test the occurrence of a direct interaction of the p85 subunit of PI3-kinase with the IL-1RI/IL-1RAcP complex in cultured AH neurons, antibodies against p85 and IL-1RI were used to coimmunoprecipitate their respective antigens. There was a basal level of p85/IL-1RI interaction in the absence of stimulation, which was increased after stimulation of the neurons with IL-1β (Fig. 4*A*). The physical interaction of these two proteins was confirmed by using anti-IL-1RI antibody on p85 precipitates (Fig. 4*B*). Interaction of p85 with IL-1RI was shown to occur through the phosphorylation of a tyrosine residue in the C-terminal portion of IL-1RI (25, 27,

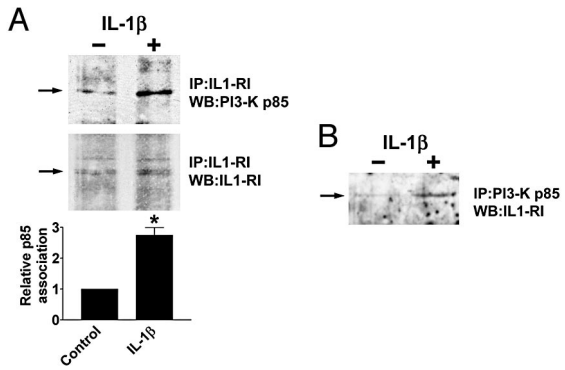

Fig. 4. IL-1β exposure increased the association of the p85 subunit of PI3-kinase to IL-1RI. Wild-type AH neurons cultured on IL-1RI$^{-/-}$ glia were incubated with 10–12 nM IL-1β for 10 min. (*A*) Immunoprecipitations were performed with 750–1,000 μg of total protein by using a polyclonal anti-IL-1RI receptor antibody, and blots were exposed to a monoclonal anti-p85 antibody (described in *Methods*). Blots were stripped, and immunoprecipitated proteins were redetected with monoclonal anti-IL-1RI antibody (described in *Methods*). Relative p85 association was calculated by normalizing p85 staining to IL-1RI staining and comparing relative to control. Representative Western blots are shown, and bar graphs indicate means ± SEM from three independent experiments. Statistical significance was determined by ANOVA followed by Tukey's test, and significance is indicated with an * compared with control at $P < 0.05$. (*B*) Immunoprecipitations were performed with 500 μg of total protein by using an anti-p85 antibody, and IL-1RI receptor association was determined by probing the blot with anti-IL-1RI antibody (described in *Methods*). The representative Western blot is shown from two independent experiments.

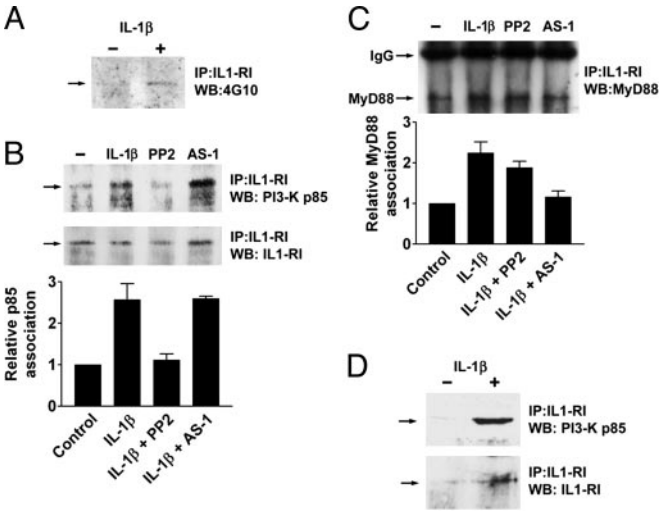

Fig. 5. The IL-1RI is tyrosine-phosphorylated, and the association of p85 subunits to IL-1RI is prevented by the Src inhibitor PP2 but not the MyD88 mimetic AS-1. (*A–C*) Cultured AH neurons from wild-type mice were developed on a preformed bed of glia from IL-1RI$^{-/-}$ mice. (*D*) Cultured AH neurons from MyD88$^{-/-}$ mice were developed on a preformed bed of glia from IL-1RI$^{-/-}$ mice. Immunoprecipitations were performed with 750–1,000 μg of total protein. (*A*) Cells were incubated with 10–12 nM IL-1β for 10 min, and the blot was probed with anti-phosphotyrosine antibody (described in *Methods*). The representative Western blot is shown from three independent experiments. (*B*) Wild-type AH cells were incubated with 10–12 nM IL-1β for 10 min after a 15-min exposure to the MyD88 mimetic AS-1 (100 μM) or the Src-family inhibitor PP2 (2 μM). Blots were stripped, and immunoprecipitated proteins were redetected with appropriate antibody, as described in *Methods*. Relative p85 association was calculated by normalizing p85 staining to IL-1RI staining and comparing relative to control. The representative Western blot is shown, and the bar graph indicates average relative p85 association (±SEM) from two independent experiments. (*C*) Lower portion of Western blots described above in *B* were probed with anti-MyD88 antibody (described in *Methods*). Relative MyD88 association was calculated by normalizing MyD88 staining to IL-1RI staining and comparing relative to control. The representative Western blot is shown, and the bar graph indicates the average relative MyD88 association (±SEM) from two independent experiments. (*D*) MyD88 $^{-/-}$ cells were incubated with 10–12 nM IL-1β for 10 min, and the blot was probed with anti-p85 antibody, stripped, and reprobed with anti-IL-1RI antibody, as described in *Methods*. The representative Western blot is shown from two independent experiments.

28). Using a phosphotyrosine-specific antibody (4G10; Upstate Biotechnology, Lake Placid, NY), we confirmed the presence of a phosphorylated tyrosine residue in IL-1RI immunoprecipitates (Fig. 5*A*).

We next tested the ability of the Src-family inhibitor PP2 and the TIR mimetic AS-1 to block the association of p85 with IL-1RI in AH cultures. The increase in p85 association with IL-1RI as a result of IL-1β treatment was blocked by PP2 but not by the addition of the MyD88 mimetic (Fig. 5*B*). When the same membranes were stripped and probed with an anti-MyD88 antibody, however, the physical interaction of MyD88 and IL-1RI was blocked by the addition of AS-1, the TIR mimetic, but not by the Src family inhibitor PP2 (Fig. 5*C*). To confirm that MyD88 was not involved in a direct interaction of the p85 subunit of PI3-kinase with the IL-1RI/IL-1RAcP complex, immunoprecipitations were performed on neurons cultured from MyD88$^{-/-}$ mice. There was a basal level of p85/IL-1RI interaction in the absence of stimulation, which was increased after stimulation of the neurons with IL-1β (Fig. 5*D*). These data confirm that p85 association and subsequent PI3-kinase activation does not depend on an interaction of MyD88 with IL-1RI in cultured AH neurons.

Discussion

The IL-1β actions in the brain involve the transcription-dependent induction of COX2, inducible NO synthase, and IL-6, which contribute to the slower inflammatory and febrile responses (1). Although some transcriptional changes, such as LPS induction of cytokines in macrophages and lymphocytes may occur within 10–15 min (29), most fast signaling involves receptor-mediated activation of signaling molecules. In our experimental system of AH neurons, IL-1RI-mediated transcription of IL-6 and IL-1 is not activated within the first 15 min (data not shown). The faster neuronal actions of IL-1β do not appear to involve transcriptional changes but rather to involve the activation of a set of protein kinases leading to posttranslational modifications of proteins, such as the IL-1RI itself, and of multiple ion channels contributing to rapid effects on neuronal activity. We show here that IL-1β induces the rapid activation of the protein tyrosine kinase Src and the Ser/Thr kinase Akt. The activation of these two kinase signaling pathways is differentially regulated in AH neurons.

The earlier synthesis of the first TIR/BB-loop mimetic AS-1 (16) was followed by the synthesis of two bifunctional TIR mimetics, EM77 and EM110. These bifunctional reagents may have an advantage over the monofunctional AS-1 given that TIR–TIR interactions involve two homotypic sites. Mutagenesis studies of the rather large TIR domain in different Toll receptors have shown that TIR–TIR interactions may use different motifs of the engaged TIR domains depending on the receptor and adaptor protein studied (30–33). This finding suggests that additional specificity of downstream blockade of Toll receptor signaling may be achieved by ''heterobifunctional'' TIR mimetics.

With the use of the bifunctional blockers EM77 and EM110 of the TIR–TIR domain interaction between the IL-1RI/IL-1RAcP and MyD88, we have demonstrated a bifurcation in IL-1β-induced fast, transcription-independent signaling. The activation of Akt is independent of the IL-1RI/MyD88 interaction, whereas the activation of Src depends on the IL-1RI/MyD88 interaction (Fig. 2 *A* and *B*). In addition, using cultured neurons from MyD88 knockout mice, we show that the Akt signaling pathway is stimulated by IL-1β in the absence of MyD88 protein, confirming the pharmacological findings with the bifunctional TIR mimetics (Fig. 2 *C* and *D*). Our data are consistent with earlier experiments showing that the IL-1-induced signaling pathways that lead to NF-κB and c-Jun N-terminal kinase activation diverge at the level of IL-1 receptor-associated kinase (34). It is possible that the mechanisms leading to the MyD88-independent activation of Akt are the same as or similar to those leading to IL-1 receptor-associated kinase-independent activation of c-Jun N-terminal kinase. Our data provide evidence of a hypothalamic neuronal association of the regulatory subunit of PI3-kinase and the IL-1R signaling complex (Fig. 4). The SH2 domains of p85 have been shown to recognize and associate with the phosphotyrosine-containing YXXM or YVXV motifs. It has been demonstrated that IL-1β-induced activation involves a physical interaction between p85 and IL-1RI (25, 27, 28). In addition to its interaction with the receptor, p85 has been shown to physically interact with the cytoplasmic domain of the IL-1RAcP in nonexcitable cells (19, 35).

IL-1β is known to initiate intracellular signaling cascades that, through the MyD88-dependent activation of NF-κB, induce the transcription of many proinflammatory genes. Although the CNS disorders follow various cellular and molecular pathologies, IL-1β appears to be a common link in many of the processes leading to neuronal death, and blockade of endogenous IL-1β has yielded neuroprotective effects (ref. 36 and reviewed in ref. 37). Delayed neuronal damage is believed to result from release of endogenous factors in response to the primary injury, among which IL-1β plays a crucial role (38). In this context, compounds blocking the IL-1RI/MyD88 interaction, such as the MyD88 mimetics described here, should prove neuroprotective by blocking both transcription-dependent and -independent inflammatory actions such as COX2 induction and Src activation, respectively. Furthermore, substantial evidence suggests a protective role for the activation of the PI3-kinase/Akt pathway in neurons (39). The IL-1β-induced Akt/PI3-kinase phosphorylation of sodium and potassium channels has been shown to be involved in the neuroprotective effects of IL-1β (39). Thus, the MyD88 mimetics described in this article offer a possible dual neuroprotective capacity: a blockade of MyD88-dependent synthesis of proinflammatory agents together with the activation of the neuroprotective PI3-kinase/Akt pathway.

The development of TIR–TIR mimetics to block Toll receptor signaling and the IL-1 signaling shown here has implications for the context of antiinflammatory drug design. The blockers of Toll-like receptor signaling (IL-1Ra; Kinneret and the IL-1β antibody) used in humans are proteins acting at extracellular sites. These proteins are delivered i.p. or s.c., not orally. More importantly, these proteins are not acting directly in the brain because the blood–brain barrier prevents their entry; thus, their use in stroke, neurodegeneration, and neuroinflammation is limited. The compounds described here are small molecules and can be applied systemically (16), promising a therapeutic advantage in several infectious and inflammatory diseases that show enhanced Toll-like receptor signaling in the periphery as well as in the brain. The blockade of TIR–TIR interactions between various members of the Toll-like receptor family and members of the MyD88, Mal, or Trif adaptor protein family provides opportunities to draw on the highly conserved nature of the TIR domain. The use of bifunctional TIR domain mimetics EM77 and EM110 over a monomeric TIR domain mimetic, such as the first BB-loop mimetic AS-1, is a step in this direction. The use of bifunctional rather than monofunctional blockers of protein–protein interactions has been fruitful (40). The number of x-ray structures of TIR domains is increasing and should accelerate and support the design process (41).

Methods

Synthesis of TIR/BB-Loop Mimetics. ***Synthesis of 2.*** A solution of 1 (13.0 g; 48.14 mmol) (16) in trifluoroacetic acid/CH_2Cl_2 (1:4, 250 ml) was stirred at room temperature for 6 h. The solvent was evaporated to dryness, and the resulting pale yellow trifluoroacetic salt (2.0 g; 7.0 mmol) was exposed to K_2CO_3 (3.87 g; 28.0 mmol) and 1-(3-bromopropyl)benzene (0.80 ml; 5.26 mmol) dimethylformamide (10 ml). The reaction mixture was heated at 80°C for 12 h and then was diluted with H_2O and extracted with Et_2O (3 times). The extracts were combined, washed with H_2O and brine, dried over $MgSO_4$, evaporated under reduced pressure, and purified by using silica gel chromatography (hexane/AcOEt mixtures) yielding 1.15 g (76%) of amine **2** as a colorless oil. ^{1}H NMR: (600 MHz, $CDCl_3$) δ 7.26 (m, 2H), 7.17 (m, 3H), 3.56 (m, 1H), 3.46 (m, 1H), 3.42 (m, 2H), 3.00 (d, J = 6.7 Hz, 1H), 2.72–2.58 (m, 3H), 2.36 (m, 1H), 2.00 (bs, 1H, NH), 1.93 (m, 2H), 1.86–1.74 (m, 5H), 0.99 (d, J = 6.7 Hz, 3H), 0.95 (d, J = 6.7 Hz, 3H); ^{13}C NMR: (150 MHz, $CDCl_3$) δ 174.2, 142.8, 128.8 (2C), 128.6 (2C), 126.0, 66.0, 48.3, 46.8, 45.9, 33.8, 32.4, 32.0, 26.6, 24.6, 20.2, 19.0.

Synthesis of 3. To a solution of 2 (600 mg; 2.07 mmol) in dry CH_2Cl_2 (30 ml) was added Et_3N (291 μl; 2.07 mmol) followed by terephthaloyl dichloride (192 mg; 0.93 mmol). The reaction mixture was stirred at ambient temperature overnight. The solvent was evaporated, and the residue was purified by silica gel chromatography (hexane/AcOEt mixtures) yielding compound **3** (330 mg; 30% yield) as a white solid. ^{1}H NMR: (600 MHz, $CDCl_3$) δ 7.33 (s, 4H), 7.20 (m, 4H), 7.11 (m, 2H), 6.88 (m, 4H),

5.23 (d, J = 10.8 Hz, 2H), 3.70 (m, 4H), 3.59 (m, 2H), 3.43 (m, 2H), 3.33 (m, 4H), 2.49 (m, 2H), 2.36 (m, 2H), 2.22 (m, 2H), 2.01 (m, 2H), 1.96 (m, 2H), 1.90 (m, 4H), 1.63 (m, 2H), 1.57 (m, 2H), 1.04 (d, J = 6.0 Hz, 12H); ^{13}C NMR: (150 MHz, $CDCl_3$) δ 171.8 (2C), 168.7 (2C), 141.0 (2C), 138.3 (2C), 128.8 (4C), 128.4 (4C), 126.7 (4C), 126.2 (2C), 60.3 (2C), 47.1 (2C), 46.4 (2C), 45.3 (2C), 33.5 (2C), 31.6 (2C), 28.3 (2C), 26.6 (2C), 24.6 (2C), 20.4 (2C), 18.8 (2C); MS: [electrospray ionization (ESI)-TOF] MH^+ calculated: 707.4536, found: 707.4521.

Synthesis of 4. To a stirred solution of 2,5-pyridine dicarboxylic acid (52 mg; 0.3 mmol) in CH_2Cl_2 (0.5 ml) and dimethylformamide (10 μl) thionyl chloride (0.4 ml) was added drop-wise at 0°C. The mixture was then heated at 40°C for 1 h, and the solvent was removed under reduced pressure. The diacid chloride obtained was added to a solution of amine **2** (200 mg; 0.69 mmol) and Et_3N (97 μl; 0.69 mmol) in dry CH_2Cl_2 (10 ml). The reaction mixture was stirred at ambient temperature overnight, the solvent was evaporated, and the residue was purified by silica gel chromatography (hexane/AcOEt mixtures) yielding compound **4** (36 mg; 17% yield) as a white solid. ^{1}H NMR: (600 MHz, $CDCl_3$) δ 8.39 (s, 1H), 7.69 (d, J = 8.1 Hz, 1H), 7.55 (d, J = 8.1 Hz, 1H), 7.20 (m, 4H), 7.11 (m, 2H), 6.94 (m, 2H), 6.86 (m, 2H), 5.21 (m, 2H), 3.70 (m, 4H), 3.59 (m, 2H), 3.43 (m, 2H), 3.31 (m, 4H), 2.51 (m, 2H), 2.40 (m, 2H), 2.29 (m, 2H), 1.98 (m, 4H), 1.90 (m, 4H), 1.68 (m, 2H), 1.54 (m, 2H), 1.04 (m, 12H); ^{13}C NMR: (150 MHz, $CDCl_3$) δ 169.6, 169.0, 168.5, 168.4, 155.6, 145.8, 141.4, 140.7, 135.1, 133.8, 128.9 (2C), 128.8 (2C), 128.5 (2C), 128.3 (2C), 126.4, 126.2, 123.6, 60.9, 60.4, 47.2, 47.0, 46.5 (2C), 45.2, 44.8, 33.6, 33.4, 31.8, 31.7, 28.3 (2C), 26.6 (2C), 24.6 (2C), 20.5, 20.3, 18.9, 18.8; MS: (ESI-TOF) MH^+ calculated: 708.4488, found: 708.4461.

The ^{1}H and ^{13}C NMR spectra data were recorded on a Bruker 600-DRX (600 MHz) spectrometer (Billerica, MA) by using solvent signals as internal references. High-resolution matrix-assisted laser desorption/ionization and high-resolution ESI-TOF spectra were acquired on an Agilent ESI-TOF mass spectrometer (Agilent Technologies, Palo Alto, CA). Crystallographic data were collected on a Bruker SMART APEX diffractometer equipped with a molybdenum sealed tube and a highly oriented graphite monochromator.

Anterior Hypothalamic Cultures. Mixed anterior hypothalamic cultures (containing neurons and glia) were prepared from Swiss–Webster mice AH. Dissociated AH from fetal mice at 13–14 days of gestation were plated at a density of one to two AH per milliliter onto preestablished astrocyte monolayers obtained from 1- to 3-day-old IL-1RI$^{-/-}$ pups while preparing mosaic cultures. The IL-1RI$^{-/-}$ mouse strain was a kind gift from Mark Labow (Hoffmann–La Roche). All neurons were cultured in MS medium (MEM with the addition of 20 mM glucose and 26.2 mM $NaHCO_3$) supplemented with 5% FBS/5% horse serum/2 mM glutamine/N2.1 supplement (GIBCO). To prevent the growth of nonneuronal cells, proliferation was halted by a 2-day exposure to 10 μM cytosine-arabinoside. Cultures were then fed every 4 days with MS plus glutamine containing 10% horse serum. Cultures were maintained in a 37°C humidified incubator in a 5% CO_2 atmosphere and used at 30–45 days *in vitro* for biochemical determinations and immunocytochemistry.

IL-1β Exposures, Immunoprecipitation, and Western Blot Analysis. Plates containing neurons and glia were washed in serum-free medium (Hepes-buffered control solution) as described in ref. 42 and equilibrated in this medium for 2–3 h at 37°C to allow recovery from the washing step. IL-1β (10–12 nM) was applied for the times indicated in the absence or presence of the MyD88 mimetic AS-1 (20–100 μM), compounds EM77 or EM110 (20 μM), the Src-family inhibitor PP2 (2 μM; Calbiochem), and the PI3-kinase inhibitor LY294002 (20 μM; Calbiochem) after which cells were washed and proteins extracted. Whole-cell extracts were separated on 10% SDS/PAGE gels and transferred to nitrocellulose membranes as described in ref. 42. For the determination of phosphorylation states of Src and Akt, membranes were incubated in a 1:1,000 dilution of phospho-Src Tyr-416 antibody (Cell Signaling Technology, Beverly, MA) or phospho-Akt Ser-473 antibody (Cell Signaling Technology) overnight at 4°C. Blots were then incubated with horseradish peroxidase-conjugated secondary antibody (Vector Laboratories) followed by chemiluminescence (Pierce). Blots were then stripped of antibodies, and the total number of proteins was determined with a 1:1,000 dilution of a pan-Src monoclonal antibody (Upstate Biotechnology) or Akt antibody (Cell Signaling Technology) for 2 h at room temperature. The ratios of phosphoproteins normalized to total staining were obtained by densitometric analysis of the bands in the films and expressed as a percentage of control (no treatment). Immunoprecipitations were performed with 750 to 1,000 μg of whole-cell extracts by using 2 μg of rabbit polyclonal anti-IL-1RI antibody (Santa Cruz Biotechnologies) or mouse monoclonal anti-PI3-kinase p85 antibody (Upstate Biotechnology). Lysates were incubated with antibody and protein G plus agarose (Santa Cruz Biotechnologies) rotating overnight at 4°C. Beads were washed three times with lysis buffer and three times with ice-cold PBS. Immunoprecipitates were resolved in 8% SDS/PAGE gels and transferred as described. Phosphorylation of PI3-kinase p85 was determined with mouse monoclonal antibody, and the IL-1RI receptor was detected with rat monoclonal antibody (R & D Systems). Tyrosine phosphorylation was determined with 1:1,000 mouse monoclonal anti-phosphotyrosine biotin-labeled antibody (clone 4G10; Upstate Biotechnology). Anti-biotin secondary antibody (Upstate Biotechnology) was used to visualize anti-phosphotyrosine proteins. To determine the extent of MyD88 association with IL-1RI in the absence or presence of AS-1 or PP2, the Western blots were cut just above the IgG heavy chain, and the top half was probed as described above. The bottom half was probed with 1:1,000 rabbit polyclonal anti-MyD88 antibody (Chemicon) redetected as above.

We thank the Skaggs Institute for support and Dr. R. Chadha for the x-ray structures. E.M. is grateful for a postdoctoral fellowship from the Spanish Ministerio de Educacion y Ciencia (Secretaria de Estado de Universidades e Investigacion). This study was supported by National Institutes of Health Grant R01 NS043501 (to T.B.). The Scripps manuscript no. is 17954-MIND.

1. Dinarello, C. A. (2004) *J. Endotoxin Res.* **10,** 201–222.
2. Dinarello, C. A. (2000) *Chest* **118,** 503–508.
3. Alam, M. N., McGinty, D., Bashir, T., Kumar, S., Imeri, L., Opp, M. R. & Szymusiak, R. (2004) *Eur. J. Neurosci.* **20,** 207–216.
4. Manfridi, A., Brambilla, D., Bianchi, S., Mariotti, M., Opp, M. R. & Imeri, L. (2003) *Eur. J. Neurosci.* **18,** 1041–1049.
5. De Sarro, G., Gareri, P., Sinopoli, V. A., David, E. & Rotiroti, D. (1997) *Life Sci.* **60,** 555–564.
6. Allan, S. M., Tyrrell, P. J. & Rothwell, N. J. (2005) *Nat. Rev. Immunol.* **5,** 629–640.
7. Ballou, L. R., Chao, C. P., Holness, M. A., Barker, S. C. & Raghow, R. (1992) *J. Biol. Chem.* **267,** 20044–20050.
8. Dunne, A. & O'Neill, L. A. (2003) *Science STKE* **171,** re3.
9. Akira, S. & Takeda, K. (2004) *Nat. Rev. Immunol.* **4,** 499–511.
10. Beutler, B. (2003) *Annu. Rev. Pharmacol. Toxicol.* **43,** 609–628.
11. Li, X. & Qin, J. (2005) *J. Mol. Med.* **83,** 258–266.
12. Burns, K., Clatworthy, J., Martin, L., Martinon, F., Plumpton, C., Maschera, B., Lewis, A., Ray, K., Tschopp, J. & Volpe, F. (2000) *Nat. Cell Biol.* **2,** 346–351.
13. Bird, T. A., Kyriakis, J. M., Tyshler, L., Gayle, M., Milne, A. & Virca, G. D. (1994) *J. Biol. Chem.* **269,** 31836–31844.
14. Dinarello, C. A., Gatti, S. & Bartfai, T. (1999) *Curr. Biol.* **9,** R147–R150.
15. O'Neill, L. (2000) *Biochem. Soc. Trans.* **28,** 557–563.

16. Bartfai, T., Behrens, M. M., Gaidarova, S., Pemberton, J., Shivanyuk, A. & Rebek, J., Jr. (2003) *Proc. Natl. Acad. Sci. USA* **100,** 7971–7976.
17. O'Neill, L. A. (2003) *Biochem. Soc. Trans.* **31,** 643–647.
18. Beutler, B., Hoebe, K., Georgel, P., Tabeta, K. & Du, X. (2004) *Microbes Infect.* **6,** 1374–1381.
19. Sizemore, N., Leung, S. & Stark, G. R. (1999) *Mol. Cell. Biol.* **19,** 4798–4805.
20. Neumann, D., Lienenklaus, S., Rosati, O. & Martin, M. U. (2002) *Eur. J. Immunol.* **32,** 3689–3698.
21. Funakoshi-Tago, M., Tago, K., Andoh, K., Sonoda, Y., Tominaga, S. & Kasahara, T. (2005) *J. Biochem. (Tokyo)* **137,** 189–197.
22. Adachi, O., Kawai, T., Takeda, K., Matsumoto, M., Tsutsui, H., Sakagami, M., Nakanishi, K. & Akira, S. (1998) *Immunity* **9,** 143–150.
23. Knight, Z. A., Chiang, G. G., Alaimo, P. J., Kenski, D. M., Ho, C. B., Coan, K., Abraham, R. T. & Shokat, K. M. (2004) *Bioorg. Med. Chem.* **12,** 4749–4759.
24. Djordjevic, S. & Driscoll, P. C. (2002) *Trends Biochem. Sci.* **27,** 426–432.
25. Reddy, S. A., Huang, J. H. & Liao, W. S. (1997) *J. Biol. Chem.* **272,** 29167–29173.
26. Madge, L. A. & Pober, J. S. (2000) *J. Biol. Chem.* **275,** 15458–15465.
27. Marmiroli, S., Bavelloni, A., Faenza, I., Sirri, A., Ognibene, A., Cenni, V., Tsukada, J., Koyama, Y., Ruzzene, M., Ferri, A., *et al.* (1998) *FEBS Lett.* **438,** 49–54.
28. Bavelloni, A., Santi, S., Sirri, A., Riccio, M., Faenza, I., Zini, N., Cecchi, S., Ferri, A., Auron, P., Maraldi, N. M. & Marmiroli, S. (1999) *J. Cell Sci.* **112,** 631–640.
29. Ojaniemi, M., Glumoff, V., Harju, K., Liljeroos, M., Vuori, K. & Hallman, M. (2003) *Eur. J. Immunol.* **33,** 597–605.
30. Li, C., Zienkiewicz, J. & Hawiger, J. (2005) *J. Biol. Chem.* **280,** 26152–26159.
31. Dunne, A., Ejdeback, M., Ludidi, P. L., O'Neill, L. A. & Gay, N. J. (2003) *J. Biol. Chem.* **278,** 41443–41451.
32. Hacker, H., Redecke, V., Blagoev, B., Kratchmarova, I., Hsu, L. C., Wang, G. G., Kamps, M. P., Raz, E., Wagner, H., Hacker, G., *et al.* (2005) *Nature* **439,** 204–207.
33. Toshchakov, V. U., Basu, S., Fenton, M. J. & Vogel, S. N. (2005) *J. Immunol.* **175,** 494–500.
34. Li, X., Commane, M., Jiang, Z. & Stark, G. R. (2001) *Proc. Natl. Acad. Sci. USA* **98,** 4461–4465.
35. Reddy, S. A., Lin, Y. F., Huang, H. J., Samanta, A. K. & Liao, W. S. (2004) *Biochem. Biophys. Res. Commun.* **316,** 1022–1028.
36. Loddick, S. A. & Rothwell, N. J. (1996) *J. Cereb. Blood Flow Metab.* **16,** 932–940.
37. Hanisch, U. K. (2002) *Glia* **40,** 140–155.
38. Rothwell, N. (2003) *Brain Behav. Immun.* **17,** 152–157.
39. Diem, R., Hobom, M., Grotsch, P., Kramer, B. & Bahr, M. (2003) *Mol. Cell. Neurosci.* **22,** 487–500.
40. Pepys, M. B., Herbert, J., Hutchinson, W. L., Tennent, G. A., Lachmann, H. J., Gallimore, J. R., Lovat, L. B., Bartfai, T., Alanine, A., Hertel, C., *et al.* (2002) *Nature* **417,** 254–259.
41. Khan, J. A., Brint, E. K., O'Neill, L. A. & Tong, L. (2004) *J. Biol. Chem.* **279,** 31664–31670.
42. Heidinger, V., Manzerra, P., Wang, X. Q., Strasser, U., Yu, S. P., Choi, D. W. & Behrens, M. M. (2002) *J. Neurosci.* **22,** 5452–5461.